In Vitro Cell. Dev. Biol.—Plant 38:168–172, March–April 2002
DOI: 10.1079/IVP2001274

1054-5476/02 $10.00+0.00

RAPID PROPAGATION OF *PHALAENOPSIS* FROM FLORAL STALK-DERIVED LEAVES

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(Received 26 April 2001; accepted 6 November 2001; editor S. Guha-Mukherjee)

Summary

An efficient and rapid *in vitro* method was developed for regeneration of *Phalaenopsis* using leaf segments derived *in vitro* from flower stalk nodes. Leaf segments of four cultivars Tinny Sunshine 'Annie', 'Taisuco Hatarot', Teipei Gold 'Golden Star', Tinny Galaxy 'Annie' cultured on Murashige and Skoog medium supplemented with N^6-benzyladenine (BA; 88.8 μM) and α-naphthaleneacetic acid (NAA; 5.4 μM) produced an average of 10–13 protocorm-like bodies (PLBs) after 12 wk. PLB proliferation was achieved on a modified Hyponex medium (1 g l^{-1} 6.5N–4.5P–19K + 20N–20P–20K + 2 g l^{-1} peptone + 3% (w/v) potato homogenate + 0.05% activated 1 g l^{-1} charcoal) and an optimal number of 13–18 PLBs developed from single PLB sections of different cultivars. Plantlet development was also achieved on a modified Hyponex medium. By repeated subculture of PLBs on a proliferation medium, and culturing them in the plantlet regeneration medium, plantlets could be produced continuously. Approximately 6 mo. were required from the initiation of culture to the production of plantlets for transplant to community pots.

Key words: orchid; propagation; protocorm-like bodies (PLBs); regeneration.

Introduction

Phalaenopsis orchid hybrids have a high market value as cut flowers and potted plants throughout the world. *Phalaenopsis* hybrids are difficult to propagate vegetatively. Characteristics are not uniform, and therefore propagation through tissue culture is desirable. Many researchers have developed *in vitro* protocols for *Phalaenopsis* (Arditti and Earnst, 1993) and recently large-scale PLB propagation using a bioreactor has been developed (Park et al., 2000). Not all genotypes of *Phalaenopsis* behave in the same manner under identical culture conditions (Reuter, 1983). However, the protocol developed by Tanaka and Sakanishi (Tanaka and Sakanishi, 1977, 1980, 1985; Tanaka, 1992) using leaves obtained from flower stalk nodes was successful and this procedure can regenerate plants which produce uniform flowers. We have followed this method for propagation of many *Phalaenopsis* hybrids that are regularly grown by orchid growers, but this method was not successful with many of the available hybrids. Therefore, we decided to refine the methodology developed by Tanaka and Sakanishi (1977, 1980, 1985) and developed an efficient and rapid propagation method. We investigated the role of growth regulators, sucrose concentration, effect of light on the induction of PLBs from leaf explants and PLB proliferation and conversion into plantlets. We report here a successful *in vitro* propagation method for *Phalaenopsis* by using leaf explants derived *in vitro* from flower stalk nodes.

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Materials and Methods

Plant materials and preparation of explants. Four popular *Phalaenopsis* hybrids, namely 'Taisuco Hatarot' (pink-flowered cultivar), Tinny Sunshine 'Annie' (white-flowered cultivar), Taipei Gold 'Golden Star' (yellow-flowered cultivar), and Tinny Galaxy 'Annie' (white flowers with purple spots) were used as source material for culture. Initially flower stalks bearing 2nd to 4th nodes from the base, all with lateral buds, were used as explants to obtain leaves. Sections (2 cm) containing buds were decontaminated for 20 min in a 3.0% sodium hypochlorite solution and then washed three times in sterilized distilled water. Explants were cultured on MS medium (Murashige and Skoog, 1962), which was taken in 25 × 250 mm culture tubes containing 20 ml of medium. The medium was supplemented with 20.2 μM N^6-benzyladenine (BA) and 45 g l^{-1} sucrose and was solidified with 7.0 g l^{-1} Phyto agar (Duchefa, Haarlem, The Netherlands). Leaves, which developed from the lateral bud region, were harvested (after 4 wk) and used as explants for further studies. The flower stalk sections were re-cultured on fresh medium for the development of new leaves.

PLB induction. The young leaves were cut into 10 × 5 mm sections and cultured on half-strength MS (1/2MS) medium (Chen et al., 2000) with BA (0, 13.3, 22.2, 44.4, 88.8, and 111 μM), α-naphthaleneacetic acid (NAA; 0, 2.7, and 5.4 μM) and 10% (v/v) coconut water. Twenty-five segments were cultured in a plastic Petri dish (90 × 15 mm) on 30 ml of medium and the abaxial surface of the leaf segments remained in contact with the medium. In another two separate experiments, various sucrose concentrations (0, 15, 30, and 45 g l^{-1}) and different light illumination (10, 30, and 60 μmol m^{-2} s^{-1}) were tested on regeneration of PLBs from leaf explants.

Proliferation of PLBs. For proliferation of PLBs, the protocorms were cut and the basal sections were cultured upside down on five different media, VW (Vacin and Went, 1949), KC (Knudson, 1946), LM (Lindemann et al., 1970), and MS and modified Hyponex (Kano, 1965; 1 g l^{-1} 6.5N–4.5P–19K + 1 g l^{-1} 20N–20P–20K) supplemented with 2 g l^{-1} peptone (Duchefa), 3% (w/v) potato homogenate and 0.05% activated charcoal (Duchefa). All media were supplemented with 30 g l^{-1} sucrose and without growth regulators. Proliferation of PLBs was tested on solid, liquid and liquid medium with a cotton raft support. In all experiments the explants were cultured in 30 ml medium in 100-ml flasks. The liquid cultures were agitated on an orbital

shaker at 50 rpm. The pH of VW, KC, LM and modified Hyponex media was adjusted to 5.2 and of MS to 5.7. All media, either liquid or solid (7 g l^{-1} Phyto agar), were autoclaved at 110 kPa for 20 min at 120°C. All cultures were incubated at 25 ± 1°C, and under cool-white fluorescent light of 30 μmol m^{-2} s^{-1} for 16 h per day.

Plantlet regeneration. PLBs were subcultured on modified Hyponex medium supplemented with 30 g l^{-1} sucrose, 2 g l^{-1} peptone + 3% (w/v) potato homogenate and 0.05% activated charcoal and solidified with 7 g l^{-1} Phyto agar for plantlet regeneration.

Experimental design and data analysis. Experiments were performed in a randomized design and repeated twice. Each treatment had 10 replicates and each replicate consisted of 25 explants per culture vessel. Morphogenetic response (PLB formation) from leaf sections was evaluated after 12 wk of culture and from PLB sections after 8 wk of culture. Morphogenetic response was expressed as percentage of explants with PLB in relation to the number of surviving explants. Average number of PLB formed per explant was also recorded. The data were statistically analyzed and means were compared using Duncan's multiple range test by the Statistical Analysis System (SAS Institute, Cary, NC; 1989).

Transplantation of in vitro *plantlets.* *In vitro*-raised plantlets (>3–4 cm height) were transplanted to pots (11 cm in size) containing peat moss and perlite (1:1) and kept in a greenhouse where humidity was maintained at 60%, temperature at 30/25°C (day/night), and light intensity at 500 μmol m^{-2} s^{-1} for 16 h per day. Plants were fertilized with Hyponex (6.5N–4.5P–19K) at 15-d intervals.

Results and Discussion

In our preliminary experiments, we tested 1/2MS, VW, KC, LM, and Hyponex media for PLB induction from leaf explants and obtained optimal results with the explants cultured on 1/2MS medium. Therefore we chose 1/2MS medium to examine the regeneration potential (PLB induction) of various cultivars using leaf explants. All regeneration experiments were conducted initially with cultivar 'Taisuco Hatarot' and subsequently the developed protocols were extended to other cultivars.

Influence of BA in combination with NAA on PLB induction. The effect of BA in combination with NAA at different concentrations was assessed on leaf cultures of cultivar 'Taisuco Hatarot' on 1/2MS medium. A lower concentration of BA (13.3 μ*M*) in combination with a lower concentration of NAA (2.7 μ*M*) resulted in no PLB production, although the explants could survive up to 12 wk of culture. With increased BA concentration and at constant NAA (2.7 and 5.4 μ*M*) PLB induction was triggered. On medium supplemented with BA (88.8 μ*M*) and NAA (5.4 μ*M*), 85% of cultures produced a maximum of 12 PLBs (Table 1; Fig. 1). The interaction between NAA and BA was significant ($P = 0.01$) for the number of PLBs regenerating on leaf explants (Table 1). The explants showed small protuberances on the surface of the explants after 4 wk. They continued to develop into PLBs over 6–8 wk (Fig. 2*A*). By the end of 12 wk, most explants were covered with PLBs (Fig. 2*B*). Tanaka and Sakanishi (1977, 1980) have used BA (10 mg l^{-1}) and NAA (1 mg l^{-1}) for regeneration of PLBs from the leaf explants and they were able to induce an average 3.8 PLBs on Hyponex medium and 1.4 PLBs on MS medium per explant. In the present study we have optimized growth regulator combinations and obtained the highest regeneration of PLBs (12 per explant) on 1/2MS medium with BA (88.8 μ*M*) and NAA (5.4 μ*M*). Similarly, NAA is used in combination with BA during *in vitro* propagation of *Aranda* (Lakshmanan et al., 1995).

Influence of sucrose concentration on PLB induction. There was no systematic effort to find the role of sucrose concentration during PLB induction in earlier experiments. Tanaka and Sakanishi (1977, 1980) and Haas-von Schmude (1983, 1985) used 30 g l^{-1} sucrose during leaf cultures of *Phalaenopsis*. In the present study, medium was supplemented with 15, 30, 45, and 60 mg l^{-1} sucrose or used without sucrose during PLB induction from leaf sections. PLBs did not develop from explants cultured on sucrose-free medium. PLBs developed on medium supplemented with sucrose and regression analysis showed that 30 mg l^{-1} sucrose is optimal for PLB induction (Fig. 3).

TABLE 1

PLB INDUCTION OF *PHALAENOPSIS* 'TAISUCO HATAROT' LEAF EXPLANTS AFTER 12 WK OF CULTURE ON MS MEDIUM WITH BA AND NAA

Treatments (μ*M*)		Mean percentage of PLB-forming explants	Mean number of PLBs per explant
BA	NAA		
0	0	0	0
13.3	2.7	0	0
22.2	2.7	36.0 d	3.3 cd
44.4	2.7	84.0 a	6.3 b
88.8	2.7	81.0 a	5.0 c
111.0	2.7	35.0 d	3.0 d
13.3	5.4	50.0 c	2.0 de
22.2	5.4	64.0 b	5.7 b
44.4	5.4	65.8 b	6.5 b
88.8	5.4	85.0 a	12.0 a
111.0	5.4	21.0 e	2.0 de
Significance[z]			
NAA			**
BA			**
NAA × BA			**

Mean *values* followed by the same *letter* are not significant at $P = 0.05$ by Duncan's multiple range test.

[z]Significance was determined by ANOVA: **means significant at $P = 0.01$.

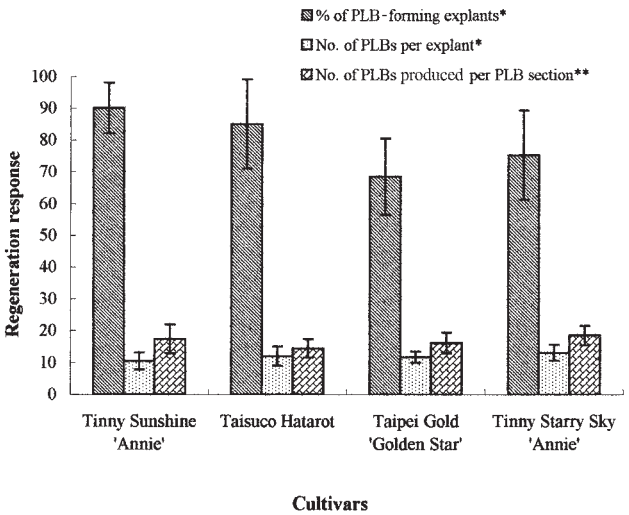

FIG. 1. Frequency of PLB induction in *Phalaenopsis* leaf and PLB section cultures. *On 1/2MS medium supplemented with BA (88.8 μ*M*) + NAA (5.4 μ*M*) + sucrose (30 g l^{-1}). **On Hyponex medium (1 g l^{-1} 6.5N–4.5P–19K + 1 g l^{-1} 20N–20P–20K) + 2 g l^{-1} peptone + 2 g l^{-1} peptone + 3% (w/v) potato homogenate + 0.05% activated charcoal + 30 g l^{-1} sucrose).

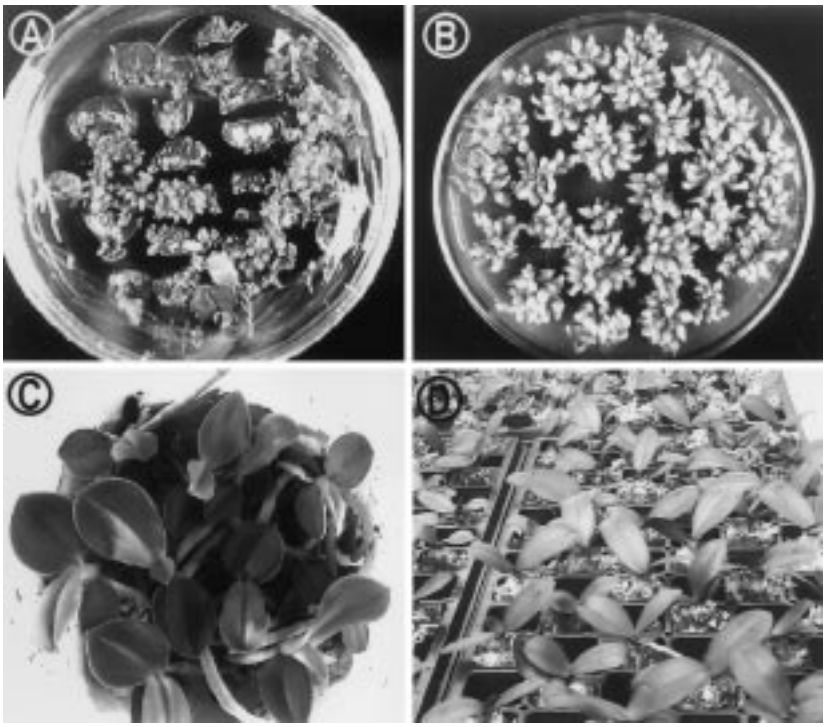

FIG. 2. *In vitro* regeneration of *Phalaenopsis* from floral stalk-derived leaves. *A*, PLBs developing from the leaf segments on $\frac{1}{2}$MS medium with BA (88.8 μM) + NAA (5.4 μM), 8 wk after culture. *B*, Well-developed PLBs on MS medium with BA (88.8 μM) + NAA (5.4 μM), 12 wk after culture. *C*, Plantlets growing on modified Hyponex (1 g l^{-1} 6.5N−4.5P−19K + 1 g l^{-1} 20N−20P−20K + 2 g l^{-1} peptone + 3% (w/v) potato homogenate + 0.05% activated charcoal + 30 g l^{-1} sucrose) medium. *D*, Acclimatized plants in greenhouse.

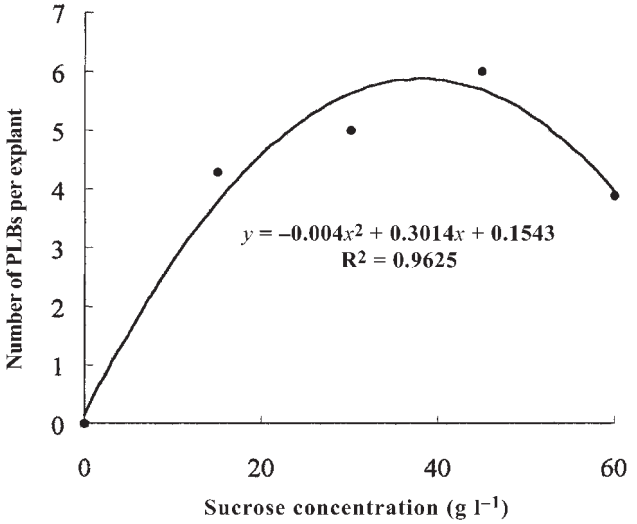

FIG. 3. Effect of sucrose concentration on PLB regeneration from leaf explants of *Phalaenopsis* 'Taisuco Hatarot' after 12 wk of culture. Leaf explants were cultured on $\frac{1}{2}$MS medium supplemented with BA (88.8 μM) + NAA (5.4 μM) + different concentrations of sucrose (0, 15, 30, and 45 g l^{-1}) and solidified with 7 g l^{-1} Phyto agar.

Influence of light intensity on PLB induction. Our earlier results show enhanced PLB regeneration from leaf section cultures when cultures were incubated under diffuse light. Tanaka and Sakanishi (1980) incubated the cultures in the dark for 2 wk and subsequently transferred to light conditions (16-h photoperiods, 900 lx) for PLB regeneration from leaf explants. Haas-von Schmude (1983, 1985) reported that PLBs form no chlorophyll in the dark but turn green and develop normally when illuminated during leaf culture of *Phalaenopsis*. To verify the appropriate light intensity suitable for explant survival and PLB induction, we have incubated cultures under light intensities of 10, 30, and 60 μmol m^{-2} s^{-1}. Low intensity of 10 μmol m^{-2} s^{-1} was best for explant survival and PLB induction (optimum of 12 PLBs per explant). An increase in light intensity to 30 and 60 μmol m^{-2} s^{-1} reduced survival percentage of the explants and PLB regeneration (Table 2).

TABLE 2

EFFECT OF LIGHT INTENSITY ON PLB FORMATION FROM LEAF SECTION EXPLANTS OF *PHALAENOPSIS* 'TAISUCO HATAROT' AFTER 12 WK IN CULTURE ON $\frac{1}{2}$MS MEDIUM (SUPPLEMENTED WITH 88.8 μM BA + 5.4 μM NAA + 30 g l^{-1} SUCROSE AND SOLIDIFIED WITH 7 g l^{-1} AGAR)

Light intensity (μmol m^{-2} s^{-1})	Mean percentage of PLB-forming explants	Number of PLBs per explant
10	90 a	12.0 a
30	85 a	10.0 ab
60	43 b	5.2 b

Mean *values* followed by the same *letter* are not significant at $P = 0.05$ by Duncan's multiple range test.

TABLE 3

EFFECT OF MURASHIGE AND SKOOG (MS), HYPONEX, VACIN AND WENT (VW), KNUDSON C (KC) AND LINDEMANN (LM) MEDIA ON SURVIVAL OF PROTOCORM EXPLANTS AND PLB PROLIFERATION OF *PHALAENOPSIS* 'TAISUCO HATAROT' AFTER 8 WK OF CULTURE

Medium	Mean percentage survival of PLBs	Mean number of PLBs developed
MS	62.2 b	18.0 a
Hyponex	75.1 a	17.4 a
VW	62.5 b	13.4 b
KC	20.0 c	4.4 c
LM	25.0 c	3.2 c

Mean *values* followed by the same *letter* are not significant at $P = 0.05$ by Duncan's multiple range test.

Hyponex medium consists of $1\,g\,l^{-1}$ 6.5N−4.5P−19K + $1\,g\,l^{-1}$ 20N−20P−20K + $2\,g\,l^{-1}$ peptone + 3% (w/v) potato homogenate + 0.05% activated charcoal.

TABLE 4

EFFECT OF CULTURE METHODS ON PROTOCORM SURVIVAL AND PLB PROLIFERATION OF *PHALAENOPSIS* 'TAISUCO HATAROT' IN HYPONEX MEDIUM AFTER 8 WK OF CULTURE

Culture method	Mean percentage survival of PLBs	Mean number of PLBs
Solid	73.5 b	17.0 a
Agitate	23.2 c	5.8 b
Raft	98.3 a	20.5 a

Mean *values* followed by the same *letter* are not significant at $P = 0.05$ by Duncan's multiple range test.

Proliferation of PLBs on five media. Tanaka and Sakanishi (1977, 1980) used solid and liquid VW media with 20% coconut water for proliferation of PLBs. Haas-von Schmude (1983, 1985) used MS medium for proliferation and differentiation of PLBs. We have tested KC, VW, MS, LM, and modified Hyponex media for proliferation and differentiation of PLBs. The explants developed PLBs in 8 wk on all the media tested. Survival percentage of the explants and multiplication of PLBs was high on MS, Hyponex, and VW media. On the other hand, survival of explants and induction of PLBs was poor on KC and LM media. The optimal explant survival was on modified Hyponex medium and an average 17.4 PLBs were produced per explant (Table 3). We tested, for the first time, raft cultures along with solid and liquid cultures for proliferation of PLBs. Raft and solid media cultures have yielded better results than liquid cultures. On rafts 98.3% of the explants were involved in multiplication with each explant producing an average 20.5 PLBs, whereas on solid medium 73.5% of explants developed 17 PLBs per explant (Table 4). Tanaka and Sakanishi (1977, 1980) used solid and liquid media cultures for proliferation of PLBs, whereas Hass-von Schmude (1983) used solid medium cultures for proliferation of PLBs. Present studies showed that raft cultures are superior to solid and liquid cultures for PLB proliferation.

Regeneration of plantlets from PLBs. PLBs were subcultured on modified Hyponex medium ($1\,g\,l^{-1}$ 6.5N−4.5P−19K + $1\,g\,l^{-1}$ 20N−20P−20K + $2\,g\,l^{-1}$ peptone + 3%(w/v) potato homogenate + 0.05% activated charcoal + $30\,g\,l^{-1}$ sucrose) upon which they developed into plantlets in 6 wk (Fig. 2*C*). However, Tanaka and Sakanishi (1985) and Tanaka (1987) used modified KC medium, and modified Hyponex medium, i.e., $3\,g\,l^{-1}$ Hyponex, Nitsch microelements, $100\,mg\,l^{-1}$ *myo*-inositol, $1\,mg\,l^{-1}$ nicotinic acid, $2\,g\,l^{-1}$ activated charcoal, $30\,g\,l^{-1}$ sucrose, and $10\,g\,l^{-1}$ Phyto agar. Reducing the production cost of *in vitro*-regenerated plants is an important factor and hence use of simple Hyponex medium during the proliferation and conversion of PLBs into plantlets is advantageous.

The leaf explants of three more cultivars, Tinny Sunshine 'Annie', Taipei Gold 'Gold Star', and Tinny Starry Sky 'Annie' were cultured on 1/2MS medium, which was optimized for PLB regeneration of cultivar 'Taisuco Hatarot' (1/2 MS supplemented with 88.8 μ*M* BA, 5.4 μ*M* NAA and $30\,g\,l^{-1}$ sucrose). About 90% of Tinny Sunshine 'Annie', 68.4% of Taipei Gold 'Gold Star', and 75.2% of Tinny Starry Sky 'Annie' leaf explants responded to cultural regimes and developed an average of 10.5, 11.7, and 13.1 PLBs per explant, respectively (Fig. 1). Subsequent culture of PLB sections of these cultivars on proliferation medium (modified Hyponex medium) showed development of an average 14.4, 16.1, and 18.5 PLBs per PLB section.

Regeneration of plantlets from PLBs. PLBs were cultured on solidified Hyponex medium ($1\,g\,l^{-1}$ 6.5N−4.5P−19N + $1\,g\,l^{-1}$ 20N−20P−20K + $2\,g\,l^{-1}$peptone + 0.05% activated charcoal + $30\,g\,l^{-1}$ sucrose) upon which they developed into plantlets in 6 wk (Fig. 2*C*).

Acclimatization. The plantlets (>3–4 cm height) were transferred to pots containing peat moss and perlite (1:1) and kept in greenhouse conditions for 2–4 wk. The plants were gradually acclimatized to greenhouse conditions (Fig. 2*D*) and the percentage survival of plants was 90%.

In conclusion, we have succeeded in increasing PLB regeneration by optimizing the culture medium (hormone and sucrose concentration) and cultural conditions (light intensity) during *Phalaenopsis* leaf section culture. We have also optimized the PLB proliferation medium (Hyponex, $1\,g\,l^{-1}$ 6.5N−4.5P−19K + $1\,g\,l^{-1}$ 20N−20P−20K + $2\,g\,l^{-1}$ peptone + 3% (w/v) potato homogenate + 0.5% activated charcoal + $30\,g\,l^{-1}$ sucrose) and method (raft culture). Furthermore, conversion of PLBs into plantlets was also achieved on the modified Hyponex medium. By repeated subculture of PLB sections on proliferation medium and harvesting of PLBs, and subsequent culture of PLBs to plantlet regeneration medium, continuous production of plants could be maintained.

Acknowledgments

This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the Research Center for the Development of Advanced Horticultural Technology at Chungbuk National University and we are grateful to Dr. E. J. Hahn for reviewing the manuscript.

References

Arditti, J.; Earnst, R. Micropropagation of orchids. New York: Wiley; 1993:467–520.

Chen, Y. C.; Chang, C.; Chang, W. C. A reliable protocol for plant regeneration from culture of *Phalaenopsis*. In Vitro Cell. Dev. Biol. Plant 36:420–423; 2000.

Haas-von Schmude, N. F. Klonale Massenvemehrung von *Phalaenopsis*. Die Orchidee 34:242–248; 1983.

Haas-von Schmude, N. F. Tissue culturing *Phalaenopsis* using leaves and leaf segments. In: Tan, K., ed. Proc. 11th Orchid Conference, Miami; 1985:311.

Kano, K. Studies on the media for orchid seed germination. Mem. Fac. Agri. Kagawa Univ. 20:1–68; 1965.

Knudson, L. A new nutrient solution for germination of orchid seed. Am. Orchid Soc. Bull. 15:214–217; 1946.

Lakshmanan, P.; Loh, C. S.; Goh, C. J. An *in vitro* method for rapid regeneration of a monopodial orchid hybrid *Aranda* Deborah using thin section culture. Plant Cell Rep. 14:510–514; 1995.

Lindemann, E. G. P.; Gunckel, J. E.; Davidson, O. W. Meristem culture of *Cattleya*. Am. Orchid Soc. Bull. 39:100–127; 1970.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.

Park, S. Y.; Murthy, H. N.; Paek, K. Y. Mass multiplication of protocorm-like bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis*. Plant Cell Tiss. Organ Cult. 63:67–72; 2000.

Reuter, E. The importance of propagating *Phalaenopsis* by tissue culture. Orchid Rev. 91:199–201; 1983.

SAS Institute. SAS/STAT user's guide, 4th edn, version 6. Cary, NC: SAS Institute; 1989.

Tanaka, M. Studies on the clonal propagation of *Phalaenopsis* through *in vitro* culture. Mem. Fac. Agric. Kagawa Univ. 49:1–85; 1987.

Tanaka, M. Micropropagation of *Phalaenopsis* spp. In: Bajaj, Y. P. S., ed. Biotechnology in agriculture and forestry, vol. 20. Berlin: Springer-Verlag; 1992:246–268.

Tanaka, M.; Sakanishi, Y. Clonal propagation of *Phalaenopsis* by leaf culture. Am. Orchid Soc. Bull. 46:733–737; 1977.

Tanaka, M.; Sakanishi, Y. Clonal propagation of *Phalaenopsis* through tissue culture. In: Kashemsanta, M. R. S., ed. Proc. 9th World Orchid Conference, Bangkok; 1980:215–221.

Tanaka, M.; Sakanishi, Y. Regenerative capacity of *in vitro* cultured leaf segments excised from mature *Phalaenopsis* plants. Bull. Univ. Osaka Prefect Ser. B. 37:1–4; 1985.

Vacin, E.; Went, F. Some pH changes in nutrient solutions. Bot. Gaz. 110:605–613; 1949.